Method for growing stem cells

This is a continuation-in-part of PCT/EP00/08247 filed August 24, 2000, the disclosure of which is incorporated herein by reference.

The present invention is related to a method for growing stem cells.

Stem cells are commonly defined as cells which exist for the lifetime of an organism and are able to undergo symmetric and/or asymmetric divisions, to give rise to further stem cells (for preservation of the stem cell pool) and to more differentiated cells with defined life-time (for organ-specific functions). Due to this unique property they are ideal vehicles for somatic gene therapy. They would maintain the transgene for the life-time of the tissue and the organism, and would carry the transgene expression into the differentiated cells. Stem cells may be totipotent (e.g. embryonic stem cells), pluripotent (e.g. hematopoietic stem cells, neural glial stem cells, hepatocyte stem cells, chondrocytic stem cells) or unipotent (e.g. keratinocytic stem cells, muscle precursor cells, tracheal epithelial precursor cells).

Stem cells are plastic, can become trans-lineaged and/or reprogrammed in different microenvironments formed by supporting cells. Both stem cells and supporting cells can be genetically processed by way of molecular breeding and/or cellular breeding, i.e. cell fusion after genetic manipulation.

Cellular breeding is defined as cell fusion after molecular manipulation. This Cellular Breeding process (Trade Mark-to-be) can be speeded up by combining the cell fusion of cell-cell with Molecular Breeding (TM) process (Maxi-gene Incl. Redwood city, California).

EP-A-0753 574 discloses a method and a composition of a culture medium are provided for obtaining ex vivo human progenitor cell expansion. The culture medium comprises human progenitor and/or stem cells, stromal cells and growth factors. The culture medium is replaced substantially continuously at a rate sufficient to maintain an effective growth environment for expansion of progenitor cells, and at least one portion of the stromal cells are transformed fibroblast cells capable of excreting at least one growth factor which directs the proliferation and/or differenciation of said human progenitor and/or stem cells.

M. Gossen et al. disclose a tight control of gene expresseion in mamalium cells by tetracycline-responsive promoters (PNAS, USA Vol. 89, 5547-5551, 1992).

Although being the aim of many research projects, it has until now being very difficult to grow stem cells, especially lineage-committed stem cells, thereby controlling expansion and differentiation of the stem cells.

The present invention provides a method for growing stem cells comprising the steps of

- providing stem cells with supporters said supporters being genetically modified in order to provide externally regulatable interactions between the supporters and the stem cells;

- applying an external signal for starting or stopping the interactions.

According to the present invention, stem cells are co-incubated with supporters. These supporters are genetically modified to allow a regulatable interaction with the stem cells. Supporters and stem cells are interchangeable upon genetic modification, processing, and interaction. These interactions between the supporters and the stem cells are externally regulatable. "Externally regulatable" means that the interaction between the supporters and the stem cells is regulated from outside of the supporters.

Preferably, the interactions are based on the secretion or display of substances. These substances, which are secreted or displayed by the supporters control the development of the stem cells. The expansion or differentiation of the stem cells is indirectly controlled by the regulatable interactions of the supporters.

Preferably as external signals may serve the addition or removal of substances, heat, light, sound, odor, taste, touch (mechanics), and/or electromagnetic waves. The only requirement is that these external signals are able to regulate the interactions between the supporters and the stem cells.

Preferably, the supporters are cells. They may be stem cells or non-stem cells. In a preferred embodiment these supporters are forming a micro-environment.

The supporting cells can be further transformed with foreign genes to express a gene product of interest e.g. a protein of the clotting cascade, insulin, enzymes, antibodies, growth factors or the like. The supporting cells can be further mutated, processed, and/or molecular, cellular bred in order to express (a) gene product(s) of interests.

It is believed that the supporters form a micro-environment thus providing cytokines and adhesion molecules as well as direct contact between the stem cells and the supporters. Suitable supporters are skin cells, tracheal and lung cells, bone marrow stroma cells, hepatic stroma cells, glial cells or tissue cells or "spore"-like stem cells.

"Spore"-like stem cells:

Somatic stem cells so far described in the literature have well published criteria using specific markers, morphology, size, biological function, etc. Besides this known kind of cells, there is another new type of somatic stem cells - "spore"-

like stem cells. They exist in every tissues examined, and also in the embryonic bodies differentiated in vitro from embryonic stem (ES) cells.

They are smaller than erythrocytes, 5 μm and less in diamter, DAPI positive. They are quienscent, i.e., non-proliferating, and they do not possess any histocompatibility antigen at this stage. They migrate in the body freely since they are small (cf. Fig. 16 and 17).

In vivo, they can be triggered into proliferation and differentiation in situ in all tissues when microenvironment allows.

In vitro, when cultured tissue cells are centrifuged using conventional cell centrifugation condition, i.e., at 1200-1500 rpm (250-350xg) for 5 minutes using a cell centrifuge, conventional cells are in the pellet fraction. The "spore" like cells remain in the supernatant. When the supernatant is seeded in a petric-dish and incubated in a CO_2 incubator at the body temperature and under low oxygen condition, they will be triggered to expand and to adherent to the petric dish, becoming visible like any conventional cells under the microscope. The low oxygen condition can be achieved either by lightening a candle in the CO_2 incubator and closing the door, or by circulating the air in the incubator with high nitrogen concentration. Upon triggering of such spore-like stem cells to landing, the further expansion of stem cells needs appropriated micro-environment with growth factors, feeder cells, as described in the text.

Suitable secreted or displayed substances are cell based growth factors, protein growth factors, interleukines.

In particular, the supporters are genetically modified with a vector, and/or a set of vectors, and/or mutation and processing, molecular and cellular breeding, comprising a gene for the substances, e.g. interleukines, protooncogenes, oncogenes, cell cycle control genes, signal transduction genes, and/or cell based growth factors and a regulatable expression system.

A preferred regulatable expression system is the regulatable tetracycline expression system.

Preferred vectors for the transformation of the supporters are the vectors selected from the group consisting of pRetro-tet-off-E6/E7, pRetro-tet-off-(tTA deleted) E6/E7, pRetro-tet-off-U19-tsA58, pRetro-tet-off-SV40Tag, pRetro-tet-off-T2, pRetro-tet-off-BCL2, pUHD15.1-ß-gal-NeoR, pUHD10.3-TGFß3, pUHD10.3-hIL3, pUHD10.3-hIL6, pUHD10.3-hFlt3-ligand, pUHD10.3-hNGF, pUHD10.3-long CNTF, pUHD 10.3-long GDNF, pUHD 10.3-hIL2, pUHD 10.3-hIL7, pUHD 10.3-hIL4, pUHD 10.3-GMCSF, pD12YCVJC-long-CNTF, pD12YCVJC-long-GDNF, pD12YCVJC-short-CNTF and pD12YCVJC-short-GDNF, as well as pRetro-tet-on- (including pRetro-tet-ART)-derivatives, other pRetro-tet-off-(including pLP-TRE2 and pLP-RevTRE)-derivatives, adenoviral-derivatives, and lentiviral-derivatives.

Details on these vectors can be found in the examples and the figures (Fig. 18-28).

Cell lines obtainable by cellular modification, and molecular and cellular breeding of cell with the vectors of the present invention are subject of the invention. Cell lines further modified using mutation, genetic processing, cellular and molecular breedings, are also subject of the present invention.

The present invention further provides a method of curing diseases by gene therapy and/or cell therapy in combination with tissue engineering when the functional expression of stem cells is helped with the engineered architecture of the tissue, which diseases are related to insufficient, lack or disorder of stem cells, by administering to patients in need thereof, supporters being genetically modified in order to provide externally regulatable interactions between the supporters and the stem cells. Furthermore, any disease related to insufficient expression or activity of a protein or enzyme can be treated by

administering supporters and/or stem cells after expansion in particular ex vivo. But it is also possible that a transformation can be performed in vivo.

Fig. 1 shows the appearance of hIL-3, depending on the addition/removal of doxycycline in vivo.

Fig. 2 shows the appearance of hIL-6 depending on the addition/removal of doxycycline in vivo (nude mice).

Fig. 3 shows the appearance of hIL-6 in scid-NOD mice.

Fig. 3 a shows the appearance of CTNF depending on the addition/removal of doxycycline in vivo (nude mice).

Fig. 3 b shows the appearance of CNTF depending on the addition/removal of doxycycline in vivo (nude and scid NOD mice).

Fig. 4 shows the cloning of growth factor genes.

Fig. 5 to 15 show photographs of various cell cultures.

Fig. 16 and 17 show "spore"-like stem cells.

Fig. 16 shows "spore"-like stem cells stained with DAPI and photographed through (a) DAPI-fiter and (b) for phase contrast. The picture was obtained by superimposing (a) and (b).

Fig. 17 shows "spore"-like cells cultured under low O_2-condition to trigger them to adhere to the bottom of the petric-dish in order to become visible in phase-contrast microscopy.

Fig. 18 to 26 show the sequences of vectors.

Fig. 27 and 28 show the construction of a vector.

The method of the present invention is further explained by the following examples:

Example 1

hIL6 containing transgenic-keratinocytic stem cells support the growth of (sister) keratinocytic stem cells in culture: In these sets of experiments, ELISA assays were performed with supernates obtained from one

hIL6 of construct teto-hIL6 containing keratinocytic stem cell clone derived from a CMV-tTA x teto-SV40 T antigen transgenic mouse, either cultured alone, or with doxycycline included in the culture for 0-12h, 25-50h. This experiment is to test whether the secretion of cytokines affects by doxycycline in culture.

hIL6 promotes the growth of CMV-tTA x teto-SV40Tag transgenic keratinocytic stem cell line drastically by increasing cell numbers. At the presence of hIL6, the growth arrest at G1 compartment is abolished, and cells continue to grow in the presence of doxycycline.

The level of hIL6 in the supernates was slightly inhibited at time point of 12h, and increase again at 50h, up to the level of the control cells, i.e., without doxycycline. Thus, hIL6-containing clone continued to secrete hIL6 despite of the fact that doxycycline was included in the culture.

The data are interpreted as follow: Engineered stem cells support the growth of sister stem cells (internally or externally) in vitro by the combination of the following two mechanisms:

(1) The hIL6 engineered keratinocytic stem cells secrete hIL6 into supernate, target and promote the growth of sister keratinocytic stem cells - an external/heterocrine mechanism.

(2) The gene product of hIL6 engineered keratinocytic stem cells, acts intracytoplasmically, and promotes the growth of itself - an internal/autocrine mechanism.

In both events, hIL6 is able to maintain skin in the keratinoytic stem cells compartment, upon the withdraw of doxycycline.

Example 2

Doxycycline-regulatable keratinocytic stem cells promote the differentiation of keratinocytic stem/precursor cells, while doxycycline-regulatable keratinocytic stem cells transgenic with hIL6 inhibits the differentiation of keratinocytic stem cells:

Keratinocyte stem cells (possesing the markers of beta-integrin 1 high, involucrin negative) from CMV-tTA-teto-SV40Tag double transgenic mice (designated H3) and keratinocytic stem/precursor cells (possessing the markers of beta-integrin 1 low, involucrin positive) isolated from inbred mice (designated MK, provided by S. Broad) are co-cultured. MK cells are induced to differentiate to mature keratinocytes by expression of mature markers and cell morpholgy. MK cells become sheets of striated, long cells with darken color, while H3 cells maintain the stem cell morphology.

When keratinocytic stem cells trangenic with teto-hIL6 (designated H3hIL6) and MK are co-cultured, MK cells differentiate less, and H3hIL6 cells maintain in the stem cell compartment. The effect can be maintained up to 6 weeks in culture. The origin of cell types in the mixing population is identified using GFP (green fluoresecent protein) inserted into H3, H3hIL6.

These experiments of morphologicall appearance by co-culture are shown in figure 5: panel A H3+MK, panel B H3 alone, panel C MK alone, panel D H3hIL6+MK, panel E H3hIL6 alone, panel F MK alone. Comparing panel D and panel A, it is shown that co-culture of H3hIL6+MK cells prevent the differentiation of MK cells (appearance of parental cell morphology), while co-culture of H3+MK cells induces the differentiation of MK cells (appearance of new cell morphology).

Example 3

Doxycycline-regulatable keratinocytic stem cells induce the differentiation of keratinocytic stem cells, while doxycycline-regulatable keratinocytic stem cells transgenic with hCNTF promote the growth of stem cells and inhibit the differentiation of keratinocytic stem/precursor cells:

The experiment is performed with keratinocytic stem cells transgenic with long JCVp-hCNTF (designated H3LC). Co-culture of H3+MK induces the differentiation of MK cells. However, co-culture of H3LC+MK prevents the differentiation of MK cells. Cell numbers of both populations are increased drastically and die quickly when depleted of nutrition and space. Thus, unlike hIL6, which promotes the survival of H3hIL6+MK cells, hCNTF promotes rapid cell division of H3LC+MK cells. The morphological appearance of cells is shown in figure 6, panel A H3+MK, panel B H3 alone, panel C MK alone, panel D H3hIL6+MK, panel E H3hIL6 alone, panel F MK alone, panel G H3LC+MK, panel H H3LC alone, panel I MK alone.

Example 4

Cytokine-secreting, doxycycline-regulatable keratinocytic stem cells influence the differentiation of keratinocytic stem/precursor cells: It is a cell-mediated event.

Soluble cytokine such as hIL6, hCNTF, or hIL3, was included in medium of the culture dish of MK+H3 cells, no obvious morphological change could be observed due to cytokines, i.e., MK+H3 showed the differentiation pattern. It is either the cytokine molecules are short-life or the anti-differentiation effect requires direct cell cell contact.

In order to distinguish these possibilities, we performed the following experiments: Cells were seeded on separated glass coverslips. Two coverslips

of different cell type were placed in the same petric dishes with distance to prevent direct contact between two coverslips. As controls, coverslips of each cell type were cultured alone. After 3 weeks, the morphological appearance of cells was evaluated. The results are shown in figure 7: panel A MK coverslip of MK+H3-GFP coculture, panel B H3-GFP coverslip of MK+H3-GFP coculture, panel C MK coverslip of MK alone, and panel D H3-GFP coverslip of H3-GFP. Comparing panel A and panel C, panel B and panel D, it is shown that co-culture of two coverslip cells in the same tissue culture dish induces the differentiation of MK cells (appearance of new cell morphology with thin long dark cells), promotes the growth, but not the differentiation of H3-GFP.

Similar experiment was performed when hIL6-secreting H3 cells are included in the culture. The results are shown in figure 8:
panel A MK coverslip of MK+H3-GFP-hIL6 coculture, panel B H3-GFP-hIL6 coverslip of MK+H3-GFP-hIL6 coculture, panel C MK coverslip of MK alone, and panel D H3-GFP-hIL6 coverslip of H3-GFP alone. Comparing panel A and panel C, panel B and panel D of this figure, and panels A of figures 7 and 8, it is shown that hIL6 secreted from H3 prevents the differentiation of MK cells (no appearance of new cell morphology). It has no obvious differentiation effect on H3.

Experiment was performed when hCNTF-secreting H3 are included in the culture as shown in figure 9:
panel A MK coverslip of MK+H3-LC coculture, panel B H3-LC coverslip of MK+H3-LC coculture, panel C MK coverslip of MK alone, and panel D H3-LC coverslip of H3-LC alone. Comparing panel A and panel C, panel B and panel D of this figure, and panels A of figures 7 and 8 and 9, it is shown that hCNTF secreting H3 does not prevent the differentiation of MK cells (appearance of new cell morphology). It promotes the growth, but not the differentiation of H3.

Thus, the influence of cytokines on the fate of keratinocytic stem cells, i.e., growth promoting vs. survival, proliferation vs. differentiation, is cell-mediated.

Example 5

Cytokine-secreting, doxycycline-regulatable mouse keratinocytic stem cells influences the differentiation of human keratinocytes:

Besides mouse MK cells, human keratinocytes (designated Sk) were tested similarly in collaboration with B. Peault in Paris. The Sk was obtained from a legal abortion with the written consence. The results are shown in figures 10-12.

Figure 10 shows:

panel A Sk coverslip of Sk+H3-GFP coculture, panel B H3-GFP coverslip of Sk+H3-GFP coculture, panel C Sk coverslip of Sk alone, and panel D H3-GFP coverslip of H3-GFP. Comparing panel A and panel C, panel B and panel D, it is shown that co-culture of two coverslip cells in the same tissue culture dish induces the differentiation of Sk cells (appearance of new cell morphology with thin long dark cells). It promotes the growth, but not the differentiation of H3-GFP.

The experiment was performed when hIL6 secreting H3 are included in the culture as shown in figure 11:

panel A Sk coverslip of Sk+H3-GFP-hIL6 coculture, panel B H3-GFP-hIL6 coverslip of Sk+H3-GFP-hIL6 coculture, panel C Sk coverslip of Sk alone, and panel D H3-GFP-hIL6 coverslip of H3-GFP alone. Comparing panel A and panel C, panel B and panel D of this figure, and panels A of figures 11 and 10, it is shown that hIL6 secreting H3 cells prevent the differentiation of Sk cells (no appearance of new cell morphology), and provide no obvious effect on self.

The experiment was also performed when hCNTF-secreting H3 cells are included in the culture as shown in figure 12:
panel A Sk coverslip of Sk+H3-LC coculture, panel B H3-LC coverslip of Sk+H3-LC coculture, panel C Sk coverslip of Sk alone, and panel D H3-LC coverslip of H3-LC alone. Comparing panel A and panel C, panel B and panel D of this figure, and panels A of figures 10-12, it is shown that hCNTF secreting H3 cells do not prevent the differentiation of Sk cells (appearance of new cell morphology). They promote their own growth and apoptosis.

In addition, a control experiment was performed to test the specific effect of doxycycline-regulatable keratinocytic stem cells by replacing them with MK cells. The results are shown in figure 13:
panel A Sk coverslip of Sk+MK coculture, panel B MK coverslip of Sk+MK coculture, panel C Sk coverslip of Sk alone, and panel D MK coverslip of MK alone. Comparing panel A and panel C, panel B and panel D of this figure, it is shown that co-culture of two coverslips containing MK cells, Sk cells, respectively, in the same tissue culture dish does not have any effect on either cell types.

The data are interpreted as follow: Cytokine-engineered stem cells affect, specifically, the behaviour of self and keratinocytic stem/precursor cells of mouse and human origins, in vitro, by the combination of the following mechanisms: growth promoting, survival, and/or differentiation mechanisms.

(1) From therapeutical point of view, during the skin injuring or wound healing process, the application of keratinocytic stem cells (H3) in situ can induce the native stem/precursor cells (such as MK, Sk) of the host (sister, external) to differentiate into mature keratinocytes. hIL6 secreting-keratinocytic stem cells (H3hIL6) can induce the proliferation and survival of such native stem/precursor cells (such as MK, Sk) of the host (sister, external) and prevent the differentiation of such cells of the host. Thus it can contribute to the wound healing process.

(2) hCNTF secreting-keratinocytic stem cells (H3LC) can induce the rapid cell division of self and native stem/precursor cells (such as MK, Sk) of the host (sister, external). However, it induces also apoptosis.

Example 6

Doxycycline regulatable tracheal epithelial stem/precursor cells can induce cell fusion when co-culturing:

The co-culture experiment is performed using MK cells and tracheal epithelial stem/precursor cells (CFTR positive, surfactant [SFII] weak positive, ciliar negative, designated L14), and tracheal stem/precursor cells transgenic with cytokine, such as L14hIL3, L14LC. With different combinations, the coculture of L14 hIL3+ X-cells causes cell fusion, as demonstrated by membrane fusion forming pancake-like clusters. It is due to the fact that L14hIL3 cells produce surfactants, a group of phospholipid substances. Similarly substance for cell fusion are commercially available, i.e., lipofectamin (Life Science, Gibco), PEI, or transfection reagents from Qiagen.

Example 7

hIL3-secreting, doxycycline-regulatable mouse tracheal epithelial stem cells inhibits the differentiation of human lung cells:

An experiment to test the effect of mouse tracheal epithelial stem cells on human lung cells (designated Lg) was performed in collaboration with B. Peault in Paris. The Lg was obtained from a legal abortion with the written consent.

Lg, L14, L14hIL3 were seeded onto coverslips separately and two types of cells were co-cultured in the same petric dish for 3 weeks. The results are shown in figures 14-15.

In figure 14, it shows

panel A Lg coverslip of Lg+L14 coculture, panel B L14 coverslip of Lg+L14 coculture, panel C Lg coverslip of Lg alone, and panel D L14 coverslip of L14 alone. Comparing panel A and panel C, panel B and panel D, it is shown that co-culture of two coverslip in the same tissue culture dish induces the differentiation of Sk cells and L14 cells (appearance of new cell morphology).

The experiment was also performed when hIL3 secreting L14 cells were included in the culture as shown in figure 15:
panel A Lg coverslip of Lg+L14-hIL3 coculture, panel B L14-hIL3 coverslip of Lg+L14-hIL3 coculture, panel C Lg coverslip of Lg alone, and panel D L14-hIL3 coverslip of L14-hIL3 alone. Comparing panel A and panel C, panel B and panel D of this figure, and panels A, B of figures 14 and 15, it is shown that hIL3 secreting L14 cells prevent the differentiation of Lg and L14-hIL3 cells (no appearance of new morphology).

The application of tracheal epithelial stem/precursor cells will be to use these cells as a tool to change the specificities of existing conditional immortalized stem cells (to make it better) in events such as trans-lineage (airway to neuron, skin, muscle, liver, etc) commitement, trans-species commitement (mouse to human), specific somatic modification such as tet-off (doxycycline turn off) to tet-on (doxycycline turn on) using either classical genetic manipulation methods, molecular or cellular breeding. The application of the direct end-differentiation products of airway stem cells (surfactant) can be for treatment of lung injury, fighting against infection such as pseudomonas infection, or film of phospholipids for industrial purposes.

The further application of doxycycline regulatable airway stem cells is to be used as feeder cells to promote or to inhibit the growth of primary human airway or nasal epithelial cells from the biopsy of Cistic Fibrosis (CF) patients. Conventially, collagen sheet culture is used to grow such primary human cells for measuring chloride channels of the CFTR. However, insufficient quantity of cells is a handicape to such a diagnostic test. Doxycycline regulatable airway

epithelial stem cells can promote such cell growing to sufficient amount allowing accurate diagnosis and to screen potential therapeutic drugs for CF patients.

Example 8

Cytokine-containing keratinocytic stem cells and tracheal epithelial stem cells secrete cytokines known to support the growth of hematopoitic stem cells (HSC):

Using hIL3, hIL6, flk2/flt3Ligand to support the growth of HSC for several weeks in culture, and in comparing to that of stroma cell lines in supporting HSC, has been performed. In the literature there are many published data showing that hIL3, hIL6, flk2/flt3Ligand are essential to support the growth of HSC. These data show that these cytokines are essential in maintaining HSC in culture, and in increasing the transduction efficiency of retroviruses into HSC in the two chamber culture system where the experiments were performed and described in the literature.

Keratinocytic stem cell lines and tracheal epithelial stem cell lines were established from CMV-tTA x tetoCMVm-SV40Tag double transgenic mice (from H. Bujard and S. Efrat). Glial stem cell lines were established from tk-rtTAxtetoCMVm-SV40Tag double transgenic mice (from H. Bujard and S. Efrat).

Example 9

Stem cells are supported by cytokine-containing transgenic stem cells: in vivo using immunoincompetent mice (nu/nu mice or scid-NOD mice): It is for the purpose of somatic delivery of growth factors essential for

maintenance of human hematopoietic stem cells (HSC) in recipient hosts. The cytokines constructed shown to be functional for HSC are hIL3, hIL6, and flk2/flt3Ligand, and thus are used further for in vivo experiments (below).

Teto-hIL6, teto-hIL3 containing transgenic keratinocytic and tracheal epithelial stem cells derived from CMV-tTA x teto-CMVm-SV40Tag double transgenic mice were pre-cultured on DED (denuded dermis from human cosmetic operation) or DET (denuded trachea) and implanted subcutaneously (flip-in) into immunoincompetent mice (nude mice or scid-NOD mice).

Blood samples from such nude mice were collected from a tail vein of mice periodically. Sera were separated from blood clots. ELISA tests were performed on serum samples collected. After cytokines were demonstrated to appear in blood, such mice ingested doxycycline (200 μm/ml) included in the drinking water and blood collected at the time points indicated. As indicated in figures 1 (hIL3) and 2 (hIL-6), hIL3 and hIL6 are shown to appear in the blood reaching a significant amount (14.7 pg/ml for hIL3, 15.9 pg/ml for hIL6), and they were decreased when doxycycline was included in the drinking water. Upon removal of doxycycline, hIL3 and hIL6 were shown to increase to higher levels again (41.2 pg/ml for hIL3, and 14.5pg/ml for hIL6). Upon reingestion of doxycycline, the levels of cytokines were shown to decrease to zero. Similar data were obtained when scid-NOD mice were used as hosts.

The mice survive over the 5-6months of experiment without any sign of illness due to the implantation of engineered mouse stem cells delivering human cytokines. The pattern can be cyclic. Thus, in immuno-incompetent mice, it was shown that the secretion of cytokines such as hIL3 and hIL6 into the blood stream is regulated by doxycycline in the drinking water. Similar experiments were performed with pD12 YCVJC-long-CNTF (Fig. 2a in nude mice, Fig. 2b in nude and scid-NOD mice) and it was shown that secretion of CNTF is regulated by doxycycline in the drinking water.

In summary, the above protocol of somatic engineering of immuno-incompetent mice with regulatable delivery of growth factors has been tested and shown to be deliverable to high titers in immuno-incompetent mice. The growth of transgenic keratinocytic stem cells and tracheal epithelial cells, and the delivery of cytokines are shown to be subjected to the regulation of doxycycline (in culture of some cells, such as HETA cells but not other cells, such as a hIL6-containing keratinocytic cell line, when doxycycline is included in the medium); and in vivo when included in the drinking water.

The principle of this protocol can also apply to the support of the growth of stem cells of any kind, such as neural and glial stem cells, in immuno-incompetent mice, as a novel diagnostic tool for evaluating the preclinical and clinical protocols.

Following 6 months in vivo, L14-hIL3 cells on cell matrix (DED, TED), increase cell mass drastically. Cell mess is formed by sponge-like, white fused tissue, similar to lung structure with emulsion of surfactant. The structure suggests the in vivo environment promotes the maturation process of L14-hIL3 airway precursor cells to surfactant positive, differentiated cells.

Example 10

Establishment and commercialization of SCID-NOD-hu systems as diagnostics for growth and evaluation of self-renewal property of human neuronal and glial stem cells, clinical protocol and for drug targeting:

The keratinocytic stem cell line and tracheal epithelial stem cell line derived from CMV-tTAxtetoCMVm-SV40Tag double transgenic mice, and the neural-glial precursor cell lines derived from tk-rtTAxtetoCMVm-SV40Tag double transgenic mice are used in this type of experiment. These cell lines are inserted with cytokine constructs for the somatic delivery of neurotropic

factors essential for the survival and maintenance of human adult brain stem cells in recipient hosts. The cytokines constructed are pD12YCV-JC-driven GDNF and CNTF. The transgenic tracheal epithelial stem cells are pre-cultured on DED (denuded dermis) or DET (denuded trachea) or DEB (denuded brain) and implanted subcutaneously (flip-in) (in the head region) into nude or scid-NOD mice. The growth of transgenic keratinocytic stem cells and epithelial tracheal cells, neural-glial precursor cells, and the delivery of cytokines have been shown to be subjected to the regulation of doxycycline in culture and in vivo when included in the drinking water. The mice survive over the months of experiment without any sign of illness due to the implantation of engineered mouse cells delivering human cytokines. It was shown that the principle of the protocol works similarly in the immuno-competent mice for human neurotrophic factors. In addition, in vivo (nude mice), H3LC cells (H3 cells transgenic with long JCVpCNTF) induce the production of erythrocytes as shown by increasing hematocrite 3 fold over the control mice. They may become a drug to treat anemia.

When doxycycline is withdrawed from cultured neural-glial precursor cell line derived from tk-rtTAxtetoCMVm-SV40Tag double transgenic mice (designated Hirn-rtTA), these cells are induced to differentiate to mature type 2 astrocytes, neurons and oligodendrocytes and to secrete myeline in vitro and in vivo. In vitro, the presence of cell-based secreting cytokines such as hIL6, hIL3, CNTF is required. Scale up the production of myeline from culturing such cell line may become a therapeutic drug for repairment lost of myeline during nerve injuring and/or neuronal diseases. This neural-glial precursor cell line itself secretes neurotropic factors. Thus it has the application for neuroregeneration.

Example 11

Protocol for construction of pD12JCVPLong-CNTF plasmid

Similar strategy and construction protocols held for pD12JCVPLong-GDNF, pD12JCVPshort-CNTF, pD12JCVPshort-GDNF.

1. pD12JCVPLong vector (from E. Beck and J. Henson) was linearized upon NsiI restriction enzyme.

2. The sticky ends of the vector were filled using Klenow fragments of E. coli polymerase I.

3. Digestion of the linearized pD12JCVPLong vector with restriction enzyme XhoI.

4. After digestion, the DNA sample was subjected to gel electrophoresis in 0.8% preparative agarose gel to obtain ca. 6.3 kb DNA fragment (pDI2JCVPLong x NsiI/XhoI).

5. pBS-hCNTF-079 vector (from E. Beck) was linearized with restriction enzyme NotI.

6. The termini of the linearized pBS-hCNTF-079 vector was filled with Klenow fragment of E. coli DNA polymerase I in order to obtain the blunt-end.

7. The linearized and blunt-ended (pBS-hCNTF-079 x NotI) was digested with SahI.

8. After digestion, the DNA sample was subjected to gel electrophoresis in 0.8% preparative agarose gel and the 2469 bp DNA fragment containing CNTF gene was isolated.

9. The blunt- and sticky ended (CNTF x NotI/SahI) fragment (from step 8) was ligated with complementary blunt- and sticky-ended (pD12JCVPLong x NsiI/XhoI) (from step 4) vector.

10. VXL1-blue competent bacteria E. coli was transformed with DNA (from step 9), and ampicillin resistant clones were selected, and characterized to be correct.

Example 12

Protocol for construction of pRetro-off-E6E7 plasmid:

1. pLXSNE6E7 vector (from D. Galloway) was linearized upon EcoRI restriction enzyme digestion.

2. The sticky ends of the vector was filled using Klenow fragments of E.coli DNA polymerase I.

3. The termini of the linearized pLXSNE6E7 was ligated with synthetic adaptor (XhoI-NotI-BglII) purchased from Roth, Karlsruhe.

4. The newly adapted-[pLXSNE6E7 x NotI/BamHI] (step 3) was digested with NotI and BamHI.

5. After digestion, the DNA sample was subjeted to gel electrophoresis in 1% preparative agarose gel to obtain ca. 830 bp fragment of [E6E7 x Not/BamHI].

6. pRetro-off vector was digested with NotI and BamHI.

7. The fragment of [E6E7 x NotI/BamHI] was then inserted into the [pRetro-off vector x NotI/BamHI] (step 6).

8. XL1-blue competent bacteria E.coli were transformed with the construct from step 7. Ampicillin resistant clones were selected and characterized to be correct.

Example 13

Protocol-2 for construction of pRetro-off-U19tsA58 plasmid:

1. pZipNEOSV(x) vector (from P. Jat) was digested with BamHI restriction enzyme.

2. After digestion, the DNA sample was electrophoresed in 0.8 % preparative agarose gel to obtain ca 2.6 kb DNA fragment (U19tsA58 x BamHI).

3. pRetro-off vector was linearized with restriction enzyme BamHI.

4. The terminal of the linearized pRetro-OFF vector was dephosphoried with Shrimp Alkaline Phophatase (USB) from Amersham.

5. The fragment of (U19tsA58 x BamHI) (from step 2) was then inserted into the (pRetro-off vector x BamHI) (from step 4).

6. The XL1-blue competent bacteria E. coli was transformed with DNA (from step 5), and ampicillin resistant clones were selected, and characterized to be correct.

7. A similar protocol yields the respective pRetro-tet-on-x (including pRetro-tet-ART-x, from H. Blau) derived vectors, other pRetro-tet-off-x (including

pLP-TRE2 and pLP-RevTRE, Clontechniques) derived vectors, adeno5 viral derived (adeno5-x, from R. Gerald), and lenti-viral-x vectors, x=U19tsA58, SV40Tag, E6/E7, Bcl2, T2, and TGFbeta3.

Example 14

Protocol-3 for construction of pRetro-off-(tTA deleted)-E6/E7 plasmid:

1. pRetro-off-E6/E7 vector was digested with BamHI and EcoRI restriction enzymes to delete DNA motif coding for tTA.

2. After digestion, the DNA sample was electrophoresed in 0.8 % preparative agarose gel to obtain ca.6.3 kb DNA fragment (pRetro-off-E6/E7 x BamHI-EcoRI).

3. pRetro-off-E6/E7 x BamHI-EcoRI vector was blunt-ended with Klenow fragment of pol.I and was ligased with enzyme Ligase T7 (Bohringer-Mannheim).

4. XL1-blue competent bacteria E. coli was transformed with DNA (from step 3), and ampicillin resistant clones were selected, and characterized to be correct.

Example 15

Protocol for the construction of pUHD-transactivator vectors:

A. Transactivator, pUHD15.1-pCMV-tTA-ß-gal-neomycin plasmid:

1. pUHD15.1 (from H. Bujard) was linearized using BamHI restriction enzyme.

2. 5′ -end was dephosphorized using phosphatase, and the DNA sample was subjected to gel electrohoresis in 1% preparative agarose gel to obtain ca. 7255 bp fragment of (pUHD15.1BamHI).

3. IRES-ßgeo fragment which contains lacZ+neo (Ca 3050 bp)) was obtained from another plasmid (ptetotsA58IRESßgeo) (from H. Schoeler) using BamHI restriction enzyme digestion.

4. After digestion, the DNA sample was subjected to gel electrohoresis in 1% preparative agarose gel to obtain ca. 3050 bp fragment of (IRES-ßgeoBamHI).

5. The fragement of (IRES-ßgeoBamHI) (step 4) was then inserted into the (pUHD15.1BamHI) (step 2).

6. XL1-blue competent bacteria E. coli were transformed with the construct from step 5. Ampicillin resistant clones were selected and characterized to be correct.

Example 16

Protocol for the construction of pUHD-responder vectors:

B. Responder pUHD10.3 cytokine plasmids:

1. The multiple cloning site (MCS) of responder pUHD10.3 (from H. Bujard) was linearized using EcoRI and SacII (for hIL6), or EcoRI and BamHI (for hIL3), or EcoRI and XbaI (for TGFß3) or Eco RI (for hflt3 ligand exon 6) restriction enzymes.

2. After digestion, the individual DNA sample was subjected to gel electrophoresis in 1% preparative agarose gel to obtain ca. 3150 bp fragment of DNA.

3. Fragments of cDNA coding for hIL6 (EcoRI-SacII), hIL3 (EcoRI-BamHI), TGFß3 (EcoRI-XbaI) hflt3 ligand exon 6 (Eco RI) were obtained from the original supplier (A. Bernad, Genetic Institute, ATCC, Immunex), and individual restriction enzyme digested as indicated in the original publications.

4. After digestion, the DNA sample was subjected to gel electrohoresis in 1% preparative agarose gel to obtain ca. 600 bp fragment of hIL6 (EcoRI-SacII), ca. 475 bp fragment of hIL3 (EcoRI-BamHI). and ca. 1233 bp fragment of TGFß3 (EcoRI-XbaI), and ca. 1 000 bp fragment of hflt3 ligand exon 6 (Eco RI).

5. The fragement coding for the respective cytokine gene (step 4) was then inserted into the responder pUHD10.3 EcoRI-SacII (for hIL6), or EcoRI-BamHI (for hIL3), or EcoRI-XbaI (for TGFß3) or Eco RI (for hflt3 ligand exon 6) (step 2).

6. XL1-blue competent bacteria E. coli were transformed with the construct from step 5. Ampicillin resistant clones were selected and characterized to be correct.

7. A similar protocol yields also for hNGF (nerve growth factor), CNTF, GNDF, hIL2 , hIL7 (from W. Uckert), hGMCSF, and hIL4 (from NGVL, Univ. Michigan).

Example 17

Protocol for construction of pUHD10.3-hflt3 Ligand exon 6 plasmid:

1. pHuflt3l-exon6 (for human flt3 Ligand exon 6) vector was digested with EcoRI restriction enzyme.

2. After digestion, the DNA sample was electrophoresed in 0.8 % preparative agarose gel to obtain ca. 1 kb DNA fragment (hflt3 Ligand exon 6 x EcoRI).

3. pUHD 10.3vector was linearized with restriction enzyme EcoRI (pUHD 10.3 x EcoRI).

4. The terminal of the linearized pUHD10.3 vector was dephosphoried with Shrimp Alkaline Phosphatase (USB) from Amersham to give ca. 3150bp fragment.

5. The fragment of (hflt3 Ligand exon 6 x EcoRI) (from step 2) was then inserted into the (pUHD10.3 vector x EcoRI) (from step 4).

6. XL1-blue competent bacteria E. coli was transformed with DNA (from step 5), and ampicillin resistant clones were selected, and characterized to be correct.

Example 18

Protocol for construction of pAdeno5-TGFb3 plasmid:

1. pUHD10.3 TGFbeta 3 vector was digested with EcoRI and BamHI restriction enzymes.

2. After digestion, the DNA sample was electrophoresed in 0.8 % preparative agarose gel to obtain ca. 1.2 kb DNA fragment (TGFbeta 3 x EcoRI-BamHI).

3. pAdeno 5 (pACCMVpLpA) vector (from R. Gerald*) was linearized with restriction enzyme EcoRI and BamHI (pACCMVpLpA x EcoRI-BamHI).

4. The terminal of the linearized pACCMVpLpA vector was dephosphoried with Shrimp Alkaline Phosphatase (USB) from Amersham.

5. The fragment of 1.2 Kb TGFbeta 3 x EcoRI-BamHI (from step 2) was then inserted into the (pACCMVpLpA x EcoRI-BamHI) (from step 4).

6. XL1-blue competent bacteria E. coli was transformed with DNA (from step 5), and ampicillin resistant clones were selected, and characterized to be correct.

*A map is not included, since R. Gerald did not submit the vector sequence to the GeneBank. However, he published it in "DNA cloning - a practical approach: mammalian systems" pp. 285-307, eds. BD Hames and D. Glover, Oxford Univ. Press, 1995. He also provided a restriction map of this vector.

Example 19

Prococol for construction of pAdeno5-T2 plasmid:

1. pBabe puro T2 mutant beta-catenin vector (from F. Watt, pBabe puro T2 mutant beta-catenin vecotr was described in A. Zhu and F. Watt, Development 126: 2285-2298, 1999. A restriction map is provided.) was digested with BamHI restriction enzyme.

2. After digestion, the DNA sample was electrophoresed in 0.8 % preparative agarose gel to obtain ca. 2.2 kb DNA fragment (T2 x BamHI).

3. pAdeno 5 (pACCMVpLpA) vector (from R. Gerald, a map is not included, since R. Gerald did not submit the vector sequence to the GeneBank. However, he published it in "DNA cloning - a practical approach: mammalian systems" pp. 285-307, eds. BD Hames and D. Glover, Oxford Univ. Press, 1995. He also provided a restriction map of this vector.) was linearized with restriction enzyme BamHI (pACCMVpLpA x BamHI).

4. The terminal of the linearized pACCMVpLpA vector was dephosporied with Shrimp Alkaline Phosphatase (USB) from Amersham.

5. The fragment of 2.2Kb T2 x BamHI (from step 2) was then inserted into the (pACCMVpLpA x BamHI) (from step 4).

6. XL1-blue competent bacteria E. coli was transformed with DNA (from step 5), and ampicillin resistant clones were selected, and characterized to be correct.

Example 20

Protocol for construction of pAdeno5-U19tsA58 plasmid:

1. pRetro-off-U19tsA58 vector (see Example 13, Protocol-2) was digested with BamHI restriction enzyme.

2. After digestion, the DNA sample was electrophoresed in 0.8 % preparative agarose gel to obtain ca. 2.5 kb DNA fragment (U19tsA58 x BamHI).

3. pAdeno 5 (pACCMVpLpA) vector (from R. Gerald, A map is not included, since R. Gerald did not submit the vector sequence to the GeneBank. However, he published it in "DNA cloning - a practical approach: mammalian systems" pp. 285-307, eds. BD Hames and D. Glover, Oxford Univ. Press, 1995. He also provided a restriction map of this vector.) was linearized with restriction enzyme BamHI (pACCMVpLpA x BamHI).

4. The terminal of the linearized pACCMVpLpA vector was dephosphoried with Shrimp Alkaline Phosphatase (USB) from Amersham.

5. The fragment of 2.5 Kb U19tsA58 x BamHI (from step 2) was then inserted into the (pACCMVpLpA x BamHI) (from step 4).

6. XL1-blue competent bacteria E. coli was transformed with DNA (from step 5), and ampicillin resistant clones were selected, and characterized to be correct.

Example 21

Protocol for construction of pRetro-off-T2-catenin plasmid:

1. pBabe puro T2 mutant beta-catenin vector (from F. Watt, pBabe puro T2 mutant beta-catenin vector was described in A. Zhu and F. Watt, Development 126: 2285-2298, 1999. A restriction map is provided.) was digested with BamHI restriction enzymes.

2. After digestion, the DNA sample was electrophoresed in 0.8 % preparative agarose gel to obtain ca. 2.2 kb DNA fragment (T2 x BamHI).

3. pRetro-off vector was linearized with restriction enzyme BamHI (pRetro-off x BamHI).

4. The terminal of the linearized pRetro-off vector was dephosphoried with Shrimp Alkaline Phosphatase (USB) from Amersham.

5. The fragment of 2.2 Kb T2 x BamHI (from step 2) was then inserted into the pRetro-off x BamHI (from step 4).

6. XL1-blue competent bacteria E. coli was transformed with DNA (from step 5), and ampicillin resistant clones were selected, and characterized to be correct.

The vector systems Tet™ and RevTet™ (Clontechniques, April 2000, p. 10) yield similar results.

<u>**Example 22**</u>

Protocol for construction of pRetro-off-[tTA deleted] E6/E7 plasmid:

1. pRetro-off-E6/E7 vector (see Example 12) was digested with BamHI and EcoRI restriction enzymes to delete DNA motif coding for tTA.

2. After digestion, the DNA sample was electrophoresed in 0.8 % preparative agarose gel to obtain ca. 6.3 kb DNA fragment (pRetro-off-E6/E7 x BamHI-EcoRI).

3. pRetro-off-E6/E7 x BamHI-EcoRI vector was blunt-ended with Klenow fragment of pol.I and was ligased with enzyme Ligase T7 (Bohringer-Mannheim).

4. XL1-blue competent bacteria E. coli was transformed with DNA (from step 3), and ampicillin resistant clones were selected, and characterized to be correct.

The vector systems Tet™ and RevTet™ (Clontechniques, April 2000, p. 10) yield similar results.

Example 23

Protocol for construction of pRetro-off-Bcl2 plasmid:

1. pPBS-Bcl2 vector (from N. McCarkthy, pPBS-Bcl2 was provided by N. McCarkthy. A restriction map and the complete sequences are provided.) was digested with EcoRI restriction enzyme.

2. After digestion, the DNA sample was electrophoresed in 0.8 % preparative agarose gel to obtain ca. 1.0 kb DNA fragment (Bcl2 x EcoRI).

3. pRetro-off vector was linearized with restriction enzyme NotI. 5 (pRetro-off x NotI), and filled with Klenow fragment of pol.I.

4. The terminal of the linearized pRetro-off vector was dephosphoried with Shrimp Alkaline Phosphatase (USB) from Amersham.

5. The fragment of 1.0 Kb Bcl2 x EcoRI (from step 2) was then inserted into the pRetro-off x NotI (from step 4).

6. XL1-blue competent bacteria E. coli was transformed with DNA (from step 5), and ampicillin resistant clones were selected, and characterized to be correct.

The vector systems Tet™ and RevTet™ (Clontechniques, April 2000, p. 10) yield similar results.